Safety, immunogenicity and effect on viral rebound of HTI vaccines in early treated HIV-1 infection: a randomized, placebo-controlled phase 1 trial

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ABSTRACT

HTI is a novel HIV vaccine immunogen designed to elicit cellular immune responses to HIV targets associated with viral control in humans. The AELIX-002 trial was a randomized, placebo-controlled trial to evaluate as a primary objective the safety of a combination of DNA.HTI (D), MVA.HTI (M) and ChAdOx1.HTI (C) vaccines in 45 early-antiretroviral (ART) treated individuals (44 men, 1 woman; NCT03204617). Secondary objectives included T cell immunogenicity, effect on viral rebound and safety of an antiretroviral treatment interruption (ATI). Adverse events were mostly mild and transient. No related SAEs were observed. We show here that HTI vaccines were able to induce strong, polyfunctional and broad CD4 and CD8 T cell responses. All participants experienced detectable viral rebound during ATI, and resumed ART when plasma HIV-1 viral load reached either >100,000 copies/ml, >10,000 copies/ml for 8 consecutive weeks, or after 24 weeks of ATI. In post-hoc analyses, HTI vaccines were associated with a prolonged time off ART in vaccinees without beneficial HLA class I alleles. Plasma viral load at the end of ATI and time off ART positively correlated with vaccine-induced HTI-specific T cell responses at ART cessation. Despite limited efficacy of the vaccines in preventing viral rebound, their ability to elicit robust T cell responses towards HTI may be beneficial in combination cure strategies, which are currently being tested in clinical trials.

1.1 INTRODUCTION

Therapeutic vaccines designed to enhance HIV-specific T cell immunity have been postulated to be a key component of any HIV cure strategy[1]. Different therapeutic vaccine candidates have been shown to be safe, immunogenic, and able to induce broad and functional T and B cell immune responses[2–5]. However, no reduction in HIV-1 viral reservoirs, prevention of viral rebound, or suppressed viremia off ART have been reported in randomized, placebo-controlled trials of vaccines, given alone or in combination with latency reversing agents [5–7].

One potential reason for these suboptimal trial outcomes may have been T cell immunogen designs and the induction of virus-specific T cell responses with ineffective or insufficient antiviral activity. To overcome this, HTI (*HIVACAT T-cell Immunogen*)-based vaccines were designed to induce functional HIV-1-specific T cell responses that were associated with better viral control in more than 1,000 HIV-1 clade B and C infected individuals within a broad HLA class I and class II allele coverage [8] targeting the most vulnerable sites of HIV-1. The HTI immunogen includes 16 HIV-1 regions from Gag, Pol, Nef and Vif that induce T cell responses of high functional avidity and cross-reactivity and target regions of overall low diversity/entropy, even though these regions were not predicted by stringent conservation

algorithms but were based on human trial data [9,10]. Importantly, in independent cohorts of viremic controllers and individuals with break-through infection after being vaccinated with full-length proteins, recognition of viral protein segments covered by HTI were found to be generally subdominant but, when detected, were associated with better viral control and viral inhibition of clade-matched HIV isolates [11]. The 16 identified HIV-1 regions were assembled in a 529aa immunogen sequence (HTI) and expressed both in a plasmid DNA (DNA.HTI, D)[12] and two viral-vectored vaccines based on a modified vaccinia virus Ankara (MVA.HTI, M)[13] and a chimpanzee adenovirus (ChAdOx1.HTI, C)[14].

AELIX-002 was a Phase I, first-in-human, randomized, double-blind, placebo-controlled study, to evaluate the safety, immunogenicity and effect on viral rebound of DNA.HTI, MVA.HTI and ChAdOx1.HTI HIV-1 vaccines administered in a heterologous prime-boost regimen to 45 virally suppressed, early-treated individuals with HIV-1 infection .

1.2 RESULTS

A total of 45 participants (44 men and 1 one woman), virologically-suppressed for at least 1 year, were recruited from an existing Early-ART cohort[15]. Acute/recent infection at ART initiation was confirmed based on any of the following criteria: i) positive plasma HIV-1 RNA with negative serology, ii) positive Gag p24 antigen; iii) indeterminate Western blot; iv) absence of the p31 band in a positive Western blot in the context of a known exposure/reported acute retroviral syndrome and/or v) negative HIV antibody test <24 weeks from the 1st positive test and before starting ART. Participants were randomized 2:1 to receive vaccines or placebo. DNA.HTI or placebo were given at weeks 0, 4 and 8 and MVA.HTI or placebo were given at weeks 12 and 20. All participants completed the 1st vaccination regimen (DDDMM (n=30) or Placebo (n=15). Out of them, 42 reconsented to start a 2nd vaccination regimen after a favorable report from the safety monitoring comitte (SMC) once the last participant had reached week 32 of the follow-up. Second vaccination regimen started after a minimum of 24 weeks from last MVA.HTI or placebo vaccination. Participants received ChadOx.HTI or placebo at weeks 0, 12 and MVA.HTI or placebo at week 24. Finally, 41 participants (CCM (n=26)/Placebo (n=15)) entered an analytical treatment interruption (ATI) eight weeks after completing the last series of vaccination (CCM or placebo) **(Fig. 1)**.

Demographics: Table 1 shows baseline characteristics. ART was initiated after a median (range) of 55 (12-125) and 64 (6-140) days after the estimated date of HIV-1 acquisition in placebo and vaccine recipients, respectively. All participants were receiving an integrase strand transfer inhibitor (INSTI)-

based ART regimen at inclusion. Median (range) time with undetectable viral load at enrollment was 18 (13-56) and 27 (12-55) months, and median $CD4^+$ T cell counts (range) were 826 (549-2,156) and 727 (553-1,336) cells/mm^3 in the placebo and in the vaccine group, respectively (not significant for all parameters). Three placebo (20%) and 7 (23%) vaccine recipients expressed any HLA class I allele associated with spontaneous control of HIV replication, respectively (i.e. HLA-B*27:05, -B*57:01, -B*15:17 and/or -B*15:03). In addition, 6 (40%) placebo recipients and 9 (30%) vaccinees expressed HLA class I alleles associated with HIV disease progression (i.e. HLA-B*07:02, -B*08:01, -B*35:01/02/03, -B*53:01 and/or -B*54/55/56)[16].

Pre-ART HIV-1 viral sequencing: Full-genome deep sequencing was performed on HIV-1 viral sequences isolated within the first 4 weeks of ART initiation from 41 participants. 32/41(75%) participants had subtype B viruses. Phylogenetic distance to a reference sequence (HXB2) and the coverage by the HTI immunogen were comparable between placebo and vaccine recipients for any of the HIV-1 proteins included in the HTI immunogen (**Extended data Fig.1a-c)**. Median (range) number of pre-ART CTL escape mutants within sequences included in the HTI immunogen was 7 (2 to 11) and 5 (2 to 8) in the placebo and vaccine recipients, respectively (Mann-Whitney, *p*=0.0364, **Extended data Fig.1d**). The degree of pre-ART CTL escape in HTI-covered regions was not associated with replication fitness of the participants´ autologous virus (**Extended data Fig.1e**).

Safety: Severity and intensity of AEs were assessed by the investigator according to the Division of DAIDS table for grading the severity of adult and pediatric adverse events, Version 2.1. [March 2017]. Overall, vaccines were safe and well tolerated (Extended data Table 1). All participants reported solicited adverse events (AEs) related to vaccinations, which were mostly mild (Grade 1-2) and transient, except 1 participant who reported Grade 3 asthenia lasting <72h after the third MVA.HTI vaccination. A total of 440 related AEs were recorded during the entire vaccination phase (111 in placebo and 329 in vaccine recipients), out of which 76 and 229 occurred after placebo or DDDMM administrations and, 35 and 100 after placebo or CCM (**Supplementary Tables 1-4**). The most frequent AEs related to vaccinations were pain at the injection site and a flu-like syndrome. There were only two serious adverse events (SAEs) during the study - an episode of acute infectious gastroenteritis due to *Campylobacter jejuni* and an acute appendicitis that required hospitalization, both in vaccine recipients (**Extended data Table 2)**. No laboratory abnormalities related to vaccinations were reported.

Immunogenicity: Total HIV-1 and HTI-specific T cells were assessed by an *ex vivo* IFN-γ-detecting enzyme-linked immunosorbent spot (ELISPOT) assay. Both vaccination regimens (DDDMM and CCM) were immunogenic. Median (range) increase in the total frequencies of HTI-specific T cells from

baseline to the peak immunogenicity timepoint after the overall vaccination regimen was 100 (0 to 498) in the placebo group and 1,499 (120 to 3,150) SFC/million PBMC in the vaccine group **(**Mann-Whitney t test, *p*<0.0001, **Fig. 2a** and **Extended data Table 3).** This corresponded to an increase in HTI magnitude >2-fold in 10 (67%) and >3-fold in 1 (7%) of placebo recipients compared to 29 (97%) and 24 (80%) of vaccine recipients (Fisher Exact test, *p* 0.0117 and *p* <0.0001 respectively, **Extended data Table 3**). To determine the breadth of vaccine-induced T cell responses, PBMC obtained at study entry and after DDDMM and CCM or placebo were expanded *in vitro* and tested against individual 15mer overlapping peptides (OLP) covering the HTI immunogen (n=147). A cumulative breadth over the entire vaccination period of a median (range) of 5 (1-13) IFN-γ-producing responses to individual HTI-covered OLPs was detected in vaccinees without any specific pattern of immunodominance across the HIV subproteins covered by the HTI immunogen in contrast to 3 (1-8) and predominantly gag-specific responses in placebo recipients (Mann-Whitney t test, *p*=0.0125, **Fig 2b-c)**. Responses to HTI were already present in 31 participants (20 vaccine and 11 placebo recipients) before ART was initiated. The maximal magnitude of HTI-specific responses achieved during the intervention phase positively correlated with the magnitude of pre-ART HTI specific T cell responses (Spearman Rho=0.5343, *p*=0.0024 and Rho=0.4632, *p*=0.0147 for vaccine recipients at their peak immunogenicity timepoints after DDDMM or CCM respectively, **Extended data Fig. 2a**). Although HTI magnitude at peak immunogenicity timepoint was higher after DDDMM in vaccinees with pre-ART HTI-specific responses compared to those without any HTI detectable responses before ART initiation (median (range) of 2,203 (460 to 3,200) vs 808 (60 to 1,595) SFC/million PBMC, Mann-Whitney t test, *p*=0.0380), these differences were no longer statistically significant at ATI initiation (median (range) of 795 (165 to 2,705) vs 595 (50 to 980) SFC/million PBMC, Mann-Whitney t test, *p*=0.1012, **Extended data Fig. 2b)**. To determine whether HTI vaccination was able to shift the focus of the virus-specific T cells, the percentage of HTI-specific T-cell frequencies divided by the total HIV-1 proteome-specific T-cell frequencies was calculated at each time point. At time of ATI start, median (range) of 14% (0 to 50) versus 67% (0 to 100) of the total anti-HIV-1 T-cell response was HTI-specific in placebo and vaccine recipients, respectively **(**Mann-Whitney T test p<0.001, **Fig. 2d).**

To further characterize the vaccine-induced T cells, intracellular cytokine staining for IFN-γ, GranzymeB (GzmB), IL-2 and TNF-α was performed in samples obtained 4 weeks after the last CCM or placebo vaccination (week 28) with or without *in vitro* stimulation with 4 different peptide pools covering the HTI immunogen. T cell lineage, phenotype, activation and exhaustion surface markers were included in the panel. The results showed that HTI-specific responses, defined as the sum of the HTI-IFN-γ^+ populations for each of the four HTI peptide pool stimulations, were both CD4 and CD8 T cell-mediated (**Fig.2e**). Polyfunctionality analyses showed that, compared to placebo recipients, vaccinees had a

higher frequency of bi and three-function CD8 T cells expressing IFN-γ/GzmB or IFN-γ/GzmB/TNF-α, while CD4 T cells predominantly expressed combinations of IL-2, IFN-γ and TNF-α (**Fig. 2f**). Importantly and, despite such an intense vaccination regimen used in the study (DDDMM-CCM), T cell exhaustion markers were not increased in HTI-specific T cells in vaccinees compared to placebo recipients after completing the last series of vaccination (**Supplementary Data Table 5**).

Finally, we measured the *in vitro* antiviral capacity of $CD8^+$ T cells by a standard viral inhibition assay (VIA)[17] using autologous $CD4^+$ T cells infected with two laboratory-adapted HIV-1 strains (BaL (R5 tropic virus) and IIIB (X4 tropic virus)) as well as with the autologous HIV virus . Median (IQR) percentages of inhibition of BaL-isolate increased in the vaccine group from 46 (17; 75) at baseline to 75 (9; 88) % at the end of the intervention (Wilcoxon t test, p=0.0805), while it remained unchanged in the placebo group (34 (17; 60) % at baseline and 37 (14; 63) % at the end of the intervention, Wilcoxon t test, p=0.9153). When using IIIB viruses and participant's autologous viruses, significant changes in VIA were detected as well (Wilcoxon t test, p=0.0014 and 0.0176) in vaccinees in contrast to placebo recipients. However, absolute increases in viral inhibition capacity were of minor magnitude probably due to the high inhibition capacity against the autologous virus already present at study entry, and consistent with early treatment initiation **(Fig. 2g).**

Effect on viral rebound during an ATI: Forty-one participants (15 placebo and 26 vaccine recipients) interrupted ART and were monitored weekly for a maximum of 24 weeks. Criteria for ART resumption included a single HIV-1 plasma viral load (pVL) > 100,000 copies/ml, 8 consecutive determinations >10,000 copies/ml, two repeated $CD4^+$ cell counts <350 cells/mm^3 and/or development of a grade 3 or higher severity clinical symptoms suggestive of an acute retroviral syndrome (ARS), whichever appeared first. The ATI period partially overlapped with the first COVID-19 outbreak in Spain with a State of Alarm declared from 03/16/2020 to 06/20/2020. Risk mitigation strategies were quickly implemented during the pandemic to reduce premature withdrawals while reassuring participant's safety. ATI was overall well tolerated (**Supplementary Data Table 6**). Frequency of sexually transmitted infections (STI) in the study population was similar to those previously reported in MSM[18], but importantly was relatively lower during the ATI period than during the intervention phase of the study (7 vs 17 cases of STI/100 person/year, respectively). Viral suppression to undetectable levels was achieved by the 12^{th} week after ART resumption in all 35 participants assessed at the end of study visit.

As shown in **Fig. 3a-b**, pVL rebound (defined as pVL >50 copies/ml) was detected in all 41 participants after ART discontinuation at a median (range) time of 2 (1-6) and 3 (1-9) weeks in placebo and vaccine recipients, respectively (Mann-Whitney t test, p=0.1942). Time to pVL rebound, peak viremia, time to

peak viremia, slope of increase pVL or AUC pVL during the ATI were comparable between placebo and vaccine recipients **(Extended data Table 4).** Twenty-five (61%) participants resumed ART after 1 determination of pVL >100,000 copies/ml, and 1 (2%) participant after 8 consecutive determinations >10,000 copies/ml. Three participants (1 in the placebo and 2 in the vaccine group) showed symptoms compatible with ARS, but they were Grade 1-2 and did not lead to ART resumption. Four (9%) participants resumed ART at weeks 9, 12, 22 and 23 of ATI without reaching any pre-specified ART resumption criteria in the context of the COVID-19 pandemic (details provided in **Supplementary Data Table 7)**. Eleven (27%) participants completed 24 weeks of ATI, 7 of them with sustained pVL<2000 copies/mL. Five participants resumed ART at week 24, and the remaining 6 participants (2 placebo and 4 vaccine recipients) opted to remain off ART and entered an ATI extension protocol with monthly monitoring for up to a total of 72 weeks of ATI (NCT04385875). Four participants (1 placebo and 3 vaccine recipients) completed the ATI-extension with sustained pVL<2,000 copies/ml after 72 weeks off ART **(Extended data Fig. 3)**, and then resumed ART. Reasons for starting ART included worries about HIV transmission, previous good tolerability to ART and the burden of additional HIV prevention tools required for viremic individuals. In a post-hoc survival analysis for time off ART during the ATI, participants without any beneficial HLA class I alleles (32 of the 41 participants that entered the ATI period), 1 (8%) of the placebo and 8 (40%) of the vaccine recipients were able to remain off ART for 22 weeks (Δ 32%, 80%CI [7.6; 55.7] and 95%CI [-1.6; 64.9]; log-rank test p=0.1834 for all ATI), with pVL <2,000 copies/mL being observed in 1 placebo and 5 vaccine recipients, respectively **(Fig. 3c)**

Exploratory objetives.

Reservoir: Amplicon signal issues occurred for 6 (14%) participants (3 placebo and 3 vaccine recipients) for whom intact proviral DNA assay (IPDA) determinations were not available. Intact HIV-1 DNA represented a median (IQR) of 23 % (9;42) of the total HIV-1 DNA. Total and intact proviral HIV-1 DNA were highly correlated (Spearman Rho = 0.6673, p <0.0001 at study entry and Rho = 0.8716, p <0.0001 at ATI start). No differences in the reservoir decay were found between groups, either measured by total proviral HIV-1 DNA (21% vs 16% decay in the placebo and vaccine groups respectively, Wilcoxon t test, p=0.4291) or by IPDA (68% vs 66% decay in the placebo and vaccine groups respectively, Wilcoxon t test, p=0.7892) **(Extended data Fig.4).**

Correlate analyses: Potential immune and viral correlates associated with longer time off ART (i.e. less risk to reach ART resumption criteria of HIV-1 pVL >100,000 or consecutive HIV-1 pVL >10,000 for more than 8 weeks) were assessed in the subgroup of individuals that did not harbor any HLA class I allele associated with spontaneous HIV control. The magnitude of the HTI specific T cell response at ATI start was significantly associated withboth prolonged time off ART and with lower pVL at the end of ATI in

vaccinees (Spearman Rho 0.6469, *p* =0.0021 and Rho -0.6837, *p*=0.0009 respectively, **Fig. 4a and 4b**) but not in placebo recipients. Similarly, albeit not statistically significant, the cumulative breadth of HTI-specific responses at ATI start was associated with longer time off ART (Spearman Rho 0.4235, *p*=0.0628, **Supplementary Fig.1**). In terms of specificities within HTI, for those vaccinees remaining off ART longer than 12 weeks (n=8), we did not observe differences in the pattern of responses induced across the different HIV protein segments covered by HTI (**Supplementary Fig.1**).

As for T cell functionality, the frequency of $CD8^+$ -and to a lesser extent $CD4^+$- T cells expressing $GzmB^+$ was positively correlated with time off ART and with lower HIV-1 pVL at the end of ATI in vaccine, but not in placebo recipients (**Fig. 4c-f**). Although vaccinees showed an increased in *in vitro* viral inhibition capacity, this was not associated with any of the ATI outcomes. As for viral factors, we ruled out the possibility that pre-existing CTL escape in sequences covered by HTI immunogen and/or replication fitness of the participants´ autologous virus could have influenced the ability of vaccine-induced responses to control virus replication during ATI. Vaccine recipients that remained off ART for longer periods of time did not show any significant correlation with the number of HLA-adapted footprints in pre-ART sequences (Spearman Rho -0.0160, *p*=0.9467, **Extended data Fig. 5a**) and were able to control viruses not only with low but also with medium and high replicative capacity (**Extended data Fig. 5b)**. Levels of total or intact proviral HIV-1 DNA at ATI start were not associated with time to viral rebound or with longer time off ART (**Extended data Fig 5c-d**); however, the majority of participants that remained off ART for >12 weeks were amongst the ones with lower reservoir levels.

Finally, as distribution of time off ART was quite binary rather than continuous (≤12 or >12 weeks), univariate logistic regression models were used to identify factors that could influence length of time to ART resumption. In addition to the pre-ART pVL , most of the immune parameters measured at ATI start increased the odds of time off ART >12 weeks (e.g. HTI magnitude $\widehat{OR}$ 1.46, 95% CI [1.16; 1.99], *p*= 0.0052; frequency of HTI-specific $CD8^+$ $GzmB^+$ T cells at ATI start $\widehat{OR}$ 1.07, 95% CI [1.01; 1.14], *p*= 0.0240; **Fig. 5**). Conversely, reservoir levels were not associated with higher chances of remaining off ART in the regression model. Importantly, in a multivariate logistic regression model including most critical demographic covariates, such as pre-ART pVL and CD4/CD8 ratio at AELIX-002 entry, there was an increased probability for being off ART after 12 weeks of ATI for the vaccinees compared to placebo recipients ($\widehat{OR}$ 8.25, 95% CI [1.05; 140.36] (**Extended data Table 5**).

1.3 DISCUSSION

The double-blind, placebo-controlled, randomized AELIX-002 study demonstrated that HTI vaccines were safe, well tolerated, and able to induce strong, polyfunctional and broad CD4 and CD8 T cell responses focused on the HTI immunogen sequence. In agreement with preclinical data in NHP[19] and clinical trials in similar populations using other T-cell vaccines only[5,6], all participants showed detectable viral rebound during the ATI. However, in exploratory analyses we observed a positive efficacy signal on the ability to remain off ART during a 24-weeks ATI (i.e. to avoid reaching HIV-1 pVL of >100,000 cop/ml or >10,000 cop/ml for 8 consecutive weeks as per the protocol-defined ART resumption criteria) in vaccinees without beneficial HLA genetics compared to placebo recipients. The AELIX-002 trial is, to our knowledge, the first randomized, placebo-controlled trial testing therapeutic T cell vaccines in an early ART-treated population that shows a correlation between vaccine-induced immune responses and both, lower post rebound viremia and extended time off ART, providing an opportunity to identifying correlates of improved viral control.

The AELIX-002 trial results support the idea that induction of HIV-specific T cells is a key factor in improving post-rebound viral suppression during an ATI, while validating the design of the HTI immunogen to induce functional T cell responses to vulnerable sites of the virus. Indeed, the HTI vaccines used in AELIX-002 showed good coverage of the autologous viral sequences, despite some evidence of pre-existing CTL escape[20]. Importantly, HTI vaccination induced strong, long-lasting GzmB-secreting $CD8^{+}$T cells along with improved ability to inhibit replication of CCR5-tropic, CXCR4-tropic, and importantly, autologous HIV virus with a broad range of viral replicative fitness. Additionally, vaccine-induced responses targeted different HTI subunits, confirming that the HTI immunogen design does contain multiple T cell targets that can mediate effective HIV control ex vivo.

Studies testing a combination of TLR7 agonists and bNAbs in NHP have observed a correlation between lower pre-ART pVL in acute infection and time to viral rebound during an ATI[21]. In contrast, in AELIX-002, lower pre-ART pVL was not associated with longer time to first detectable pVL during the ATI but it was positively correlated with time off ART. Importantly, in exploratory multivariate models the association of vaccination with extended time off ART remained statistically significant, even after accounting for participant's levels of pre-ART viremia and CD4/CD8 ratio.

Different approaches have been developed to establish high-throughput assays to quantify the replication-competent viral reservoir relevant for cure-related trials, including the IPDA assay which allows measurement of genetically intact proviruses and excludes the majority of defective

proviruses[22,23]. In AELIX-002, although the intact proviral HIV-1 DNA declined preferentially over time relative to total proviruses, we did not detect differences in the reservoir decay from baseline to ATI associated with therapeutic vaccination, suggesting that such a reduction reflected natural decay curves due to early-treatment[15]. In contrast to others that have reported an association between a delay in viral rebound and lower intact proviral DNA levels after vesatolimod treatment in viremic controllers[24], we did not detect any correlation between levels of intact proviral DNA and time to viral rebound in our early-treated population. Of note, 7 (17%) participants that entered the ATI period had no detectable levels of intact HIV-1 proviruses at the time of ART cessation and yet experienced viral rebound during the ATI.

Despite the extended vaccination regimen used in AELIX-002, vaccinations were safe and well tolerated, and safety profiles were comparable to other HIV vaccines using same vector platforms both in HIV negative[25] or HIV positive individuals[2]. No serious related adverse events or laboratory abnormalities were observed after either DDDMM or CCM vaccinations, including any suspected vaccine-induced immune thrombotic thrombocytopenia (VITT) as described for ChAdOx1-vectored COVID19 vaccines[26]; although our sample size was limited to detect such rare events. Notewothy, T cell exhaustion markers were not increased in vaccinees compared to placebo recipients.

Similar to the ATI viral kinetics in the AELIX-002 trial in which all participants experienced a fast viral rebound, Okoye et al have recently shown in the NHP model that CD8+ T cells contribute to reduce the viral set point, although they were not able to prevent viral recrudescence[27]. These data suggest that HIV antigenic stimulation might be necessary to trigger an effective immune response during the ATI. This, in return has important implications on the design of ATI trials where ART ressumption criteria may need to be permissive enough to allow for such a transient viremia[28–30]. Initial peak viremia may however also be associated with risks for onward virus transmission, mutational T cell escape, reseeding of the viral reservoir, and/or excessive inflammatory responses giving rise to ARS. Therefore, it is critical to balance research objectives and the well-being of participants while considering, in collaboration with community advisory boards, effective transmission risk-reduction strategies [31]. In AELIX-002, ART resumption criteria during the ATI were well accepted among participants, as well as all transmission-risk reduction strategies implemented, which included PrEP provision to sexual partners, psychological support, and active surveillance for asymptomatic STI. Of note, the AELIX-002 study and, in particular the ATI phase, was ongoing when the first COVID-19 outbreak in Spain occurred. This severely impacted many clinical trial sites as most non-COVID-related hospital activities, including clinical research, had to be paused. Rapid establishment of a risk-mitigation plan overseen

by an external SMC during the emergency outbreak was critical to minimize the impact of the COVID-19 pandemic on the conduct of AELIX-002 , as some investigators have recommended recently[32,33].

The main limitations of our trial include the sample size that did not allow for a powered subgroup analysis in individuals without beneficial HLA genetics as well as the selected study population that limits extrapolation of our results to HIV populations other than those treated early during acute/recent HIV infection and in which, both cis-gender and transgender women are usually underrepresented. In addition, the regimen used in AELIX-002 consisted of two different vaccination regimens of DDDMM, further boosted by CCM vaccines, which overall, does not represent a clinically feasible vaccination regimen but did serve to set up an efficacy proof of concept of the HTI immunogen design. In fact, we acknowledge that the efficacy endpoint of time off ART in our study is a function of the ART resumption criteria used in the protocol and, importantly, not yet translatable into clinical practice.

Our findings strongly support the further use of HTI vaccines in simpler regimens, given alone or in combination with other immunomodulatory agents to improve their efficacy, to achieve more clinically relevant virological outcomes and to be better aligned with the most current target product profile for an HIV cure indication[34]. For instance, to avoid viral rebound, or partially curtail fast and severe viral recrudesence, and to improve the level of virus control, we and others have proposed strategies combining therapeutic vaccines with bNAbs, which at the same time may enhance suppressive capacity of vaccine-induced responses through a vaccinal effect[35–37]. In this sense, BCN03 and AELIX-003 clinical trials (NCT05208125 and NCT04364035, respectively) are currently exploring the safety and immunogenicity of a ChAdOx1.HTI/MVA.HTI vaccine regimen with a recombinant HIV-1 envelope SOSIP protein (ConM SOSIP.v7 gp140) or with a TLR7 agonist (Vesatolimod) . including an ATI with the same ART resumption criteria as in AELIX-002.

In conclusion, this first administration of a heterologous prime-boost regimen of HTI vaccines in early ART-treated individuals with HIV infection was safe and immunogenic. In exploratory analyses, AELIX-002 showed a potential signal for improved post-rebound viral control after ART discontinuation in a subset of individuals who did not already possess a beneficial HLA genotype, which requires validation in future studies. These data provide support the use of HTI vaccines as a T-cell-stimulating backbone for future combination cure strategies, with the addition of immunomodulators, bNAbs, or alternative vaccine vectors to boost their efficacy.

Acknowledgements. Special thanks to all the volunteers participating in this study for their perseverance and dedication. We especially thank the SMC members Eugenia Negredo, Magi Farré and Felipe Garcia for their commitment to oversee the risk-mitigation plan during the challenging COVID-19 pandemic. We thank Rafaela Ayen, Lucía Gómez, Cristina Ramírez and Eulalia Grau from the sample processing laboratory at IrsiCaixa, Badalona, Spain for their technical assistance throughout the study. We thank Barbara K. Felber and George N. Pavlakis from the NCI-Frederick, Maryland, US and Alison Crook from the Jenner Institute, The Nuffield Department of Medicine, University of Oxford, UK for their assistance in the construction and provision of pre-GMP starting materials of DNA.HTI, MVA.HTI and ChAdOx1.HTI under MTA contracts.

Author contributions. CB, IMG, JM and BM conceived and designed the study. LB and AL additionally contributed to the study design in further study amendments. LB, JC, CL, ML, JM, BM, FP and AR contributed with clinical development of the study. AL, ML, BO-T, FP, FPE and DS contributed with the data management and overall study coordination. TH and EW helped with IMP production. MC, SC, TE, ALL, MP and MR-U performed the experiments. YA-S, ALL, JM, BM, MRR and MN-J undertook the statistical analysis. LB, CB, JM and BM drafted the manuscript. LB, AL, IMG, DS, BC, CB, JM, BM and RP revised the manuscript critically for important intellectual content. All authors reviewed and approved the final version of the manuscript.

Competing interests. CB, BM and ALL are co-inventors of the HTI immunogen (patent application PCT/EP2013/051596). CB, BM and IMG are co-inventors of US patent Application No. 62/935,519 and US Appl. No. 62/851,546 which have relevance to the vaccine regimen used in this study. BM reports consultancy personal fees from AELIX THERAPEUTICS, S.L, as well as speakers fees from Gilead, Janssen, ViiV Healthcare, outside the submitted work. CB is co-founder, CSO and shareholder of AELIX THERAPEUTICS, S.L and serves as an advisor for Tendel Therapies, OmniScope, outside of the submitted work. MN-J is co-founder and shareholder of Nano1Health S.L, outside the scope of submitted work. IMG is a shareholder of, and acts as a consultant to, AELIX THERAPEUTICS, S.L. He is also the CMO of Orion Biotechnology, outside the scope of submitted work. JM has received research funding, consultancy fees and lecture sponsorships from and have served on advisory boards for various laboratories (MSD, Abbvie, Boehringer Ingelheim, Gilead Sciences, Viiv Healthcare, Janssen-Cilag and Bristol-Myers-Squibb). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding: This work was supported by AELIX Therapeutics S.L, and partly by grants from the Spanish Research Agency and the European Regional Development Fund under contract nr. RTC-2017-6473-1 [(AEI/FEDER, UE) / (MINECO/FEDER, UE)], the European Union's Horizon 2020 research and innovation program under grant agreement 681137-EAVI2020, NIH grant P01-AI131568 and by ISCIII PI20/01039. LB held a PhD grant from ISCIII (RH CM20/00097) during the conduct of the study.

Role of the sponsor: this study was funded by AELIX Therapeutics, SL, who participated in the design of the study, provided the IMP of the study, oversaw all safety monitoring activities, data analysis, interpretation and reviewed the manuscript.

Table 1. Study population. Demographic, clinical, and treatment characteristics of study participants at study entry (n = 45).

Demographics		Placebo n=15	Vaccine n=30	ITT Population n=45
Age, years		34 (20 - 51)	37 (23 - 57)	36 (20 - 57)
Sex at birth, male, n (%)		15 (100%)	29 (96.7%)	44 (97.8%)
BMI (kg/m^2)		22.5 (19.1 – 31.7)	22.8 (19.1 – 32.2)	22.8 (19.1 – 32.2)
Time from estimated HIV transmission to ART initiation (days)		55 (12 - 125)	64 (6 - 140)	63 (6 - 140)
Fiebig stage at ART initiation, n (%)*				
	I	1 (6.7%)	1 (3.3%)	2 (4.4%)
	II	0 (0%)	2 (6.7%)	2 (4.4%)
	III	2 (13.3%)	0 (0%)	2 (4.4%)
	IV	0 (0%)	2 (6.7%)	2 (4.4%)
	V	5 (33.3%)	19 (63.3%)	24 (53.3%)
	VI	7 (46.7%)	6 (20%)	13 (28.9%)
pVL at ART initiation, $\log_{10}$ copies/mL		4.9 (3.7 – 7)	4.7 (2.9 – 7)	4.7 (2.9 – 7)
Current ART, n (%)				
	DTG/ABC/3TC	7 (46.7%)	9 (30%)	16 (35.6%)
	EVG/c/ (TAF or TDF)/FTC	4 (26.7%)	13 (43.3%)	17 (37.8%)
	RAL + ABC/3TC	1 (6.7%)	2 (6.7%)	3 (6.7%)
	RAL + TDF/FTC	3 (20%)	6 (20%)	9 (20%)
Time with undetectable pVL (months)		18 (13 - 56)	27 (12 - 55)	24 (11 - 56)
Absolute CD4 (cells/mm^3)		826 (549 – 2,156)	727 (457 – 1,333)	745 (365 – 2,156)
Percentage CD4 (%)		39.2 (19 – 53.9)	35.4 (17.8 – 63.4)	36.3 (17.8 – 63.4)
CD4/CD8 ratio		1.1 (0.5 – 2.66)	1.02 (0.5 – 3.3)	1 (0.5 – 3.3)
Beneficial HLA alleles	Any	3 (20%)	7(23.3%)	10 (22.2%)
	B2705	1 (6.7%)	4 (13.3%)	5 (11.1%)
	B5701	2 (13.3%)	1 (3.3%)	3 (6.7%)
	B1517	0 (0%)	1 (3.3%)	1 (2.2%)
	B1503	0 (0%)	1 (3.3%)	1 (2.2%)
Past small-pox vaccination‡		1 (6.7%)	6 (20%)	7 (15.6%)
CCR5-Δ32 heterozygosity^		2 (13.3%)	3 (10%)	5 (11.1%)

Median (Min - Max) except where is specified.
**According to Fiebig, AIDS 2003.*
‡ Signs of scarification or history of vaccination reported by the volunteer.
^CCR5-Δ32 genotype was available for 15 placebo and 26 vaccine recipients (those entering the ATI).
Comparisons between study groups by two-sample t-Test or Chi-squared test when corresponding (non-significant for all variables).
BMI, body mass index; cART: combination antiretroviral therapy; pVL, HIV-1 plasma viral load;DTG, dolutegravir; ABC, abacavir; 3TC, lamivudine; EVG/c, elvitegravir/cobicistat; TAF, tenofovir alafenamide fumarate; TDF, tenofovir disoproxil fumarate.

Figure Legends

Fig 1. Trial design. a, Schematic trial design and study visits. **b,** Consolidated Standards of Reporting Trials (CONSORT) flow diagram for the trial. *HIV*: Human immunodeficiency virus, *ARV*: antiretroviral therapy, *ATI:* analytical treatment interruption, *D:* DNA.HTI, *M:* MVA.HTI, *C:* ChAdOx1.HTI, *P:* placebo.

Fig 2. Vaccine immunogenicity. a, Magnitude (sum of SFC/10^6 PBMC to HTI pools P1-P10) over the AELIX-002 study in placebo (blue) and vaccine (red) recipients over the two vaccination regimens (DDDMM/PPPPP and CCM/PPP) up to the start of the ATI period. **b,** Breadth of vaccine-elicited responses towards individual OLP spanning the entire HTI sequence in the 15 placebo and 30 vaccine recipients. Horizontal and error bars represent median and IQR, respectively and p-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. **c**, the distribution of HTI-specific responses within the different HIV-1 subproteins included in the HTI immunogen of the cumulative breadth at AELIX-002 study entry (above) and after the completion of last series of vaccinations (down) for each placebo (P1 to P15) and vaccine (V1 to V26) recipients. **d,** Average distribution of total HIV-1 T-cells according to their specificity at the indicated time points, HTI-specific responses are shown for placebo (blue) and vaccine (red) recipients, while the rest of non-HTI HIV-1 specific responses are shown in grey, and p-values correspond to comparison between the proportion of HTI-specific responses at each timepoint. Fisher's exact test is used for comparisons between groups. **e**, Proportion of HTI-specific CD4+ and CD8+ T cells secreting IFN-γ (left) or both IFN-γ and GzmB (right) after completion of last series of HTI vaccinations (DDDMM-CCM/PPPP-PPP). Median with interquartile range for the sum of IFN-γ^+ and IFN-γ^+/GzmB$^+$ for each of the four HTI peptide pool stimulations is shown. Wilcoxon-Mann-Whitney is used for comparison between placebo (n=12) and vaccine (n=20) groups. **f,** Polyfunctionality of HTI-specific CD4$^+$ and CD8$^+$ T cells was analyzed by Boolean gating. Pie charts and boxplots per treatment group (placebo n=15, vaccine n=26) illustrate relative and absolute proportion of each of the different subsets (cells producing 2, 3, or 4 cytokines), respectively. On each boxplot, the central line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to 1.5 times the interquartile range. Q-values correspond to Mann-Whitney test per row, adjusted for multiple comparisons. **g,** Changes in viral inhibition capacity to laboratory-adapted HIV-1 strains (placebo n=15, vaccine n=26) and autologous HIV-1 (placebo n=14, vaccine n=23) at study entry, after DDDMM/PPPPP and after CCM/PPP regimens for placebo (blue) and vaccine (red) recipients. Horizontal and error bars represent median and IQR, respectively and p-values correspond to comparisons between the indicated time points using the Wilcoxon signed-rank test. *SCR:* screening, *BSL:* baseline, *D:* DNA.HTI, *M:* MVA.HTI, *C:* ChAdOx1.HTI, *P:* placebo

Fig 3. Analytical treatment interruption (ATI) period. a, Individual HIV-1 pVL during the 24 weeks of ATI is shown for all placebo (blue) or vaccine (red) recipients and **b**, in those without any beneficial HLA associated with spontaneous viral control in the lower panel. Lines are interrupted on week of ART resumption. Dotted lines represent detection limit and the two different virologic threshold for ART resumption (10,000 and 100,000 HIV-1 RNA copies/ml, respectively). **c**, Proportion of participants without any beneficial HLA allele associated with spontaneous viral control in the placebo and vaccine arms remaining off ART following treatment interruption. Log-rank test is used for comparison between groups over the entire ATI period. Proportion of participants, delta and 80% Confidence Interval is shown for week 22 of ATI, before last two vaccine recipients resumed ART due to COVID-19 related reasons without fulfilling any per-protocol virological criteria. *pVL:* plasma viral load, *ART:* antiretroviral treatment.

Fig 4. Immune correlates with ATI outcomes in participants without any beneficial HLA allele. Correlation between time off ART (left panels) and HIV-1 pVL at the end of ATI at ART resumption timepoint (right panels) with HTI magnitude at ATI start (**a,b**), proportion of $CD8^+$ (**c,d**) and $CD4^+$ (**e,f**) GzmB-secreting T cells in placebo (blue) and vaccine (red) recipients. *Spearman's correlation is used. ART:* antiretroviral treatment, *pVL:* plasma viral load, *ATI:* analytical treatment interruption.

Fig 5. Univariate correlate analysis. Odds ratio and its 95%CI of time to ART resumption > 12 weeks in univariate logistic regression models (n=32 participants without beneficial alleles).

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METHODS

Study design. AELIX-002 (clinicaltrials.gov NCT03204617) enrolled 45 HIV-positive early-treated individuals at the Infectious Diseases Department of the Hospital Germans Trias i Pujol (HUGTIP), Badalona, Spain. First and last participants were recruited on July 20th 2017 and June 5th 2018 , respectively. The last study visit was conducted on March 10th 2021. AELIX-002 was a Phase I, proof of concept, first in human, randomized, double-blind, placebo-controlled study, to evaluate safety, immunogenicity and effect on viral rebound during an ATI of three novel HIV-1 vaccines (DNA.HTI (D), MVA.HTI (M) and ChAdOx1.HTI (C)) administered in a heterologous prime-boost regimen consisting of DDDMM and CCM vs placebo .

Participants had to be aged 18-65 years and have a history of triple-drug ART initiated within 6 months after estimated HIV-1 acquisition with HIV-1 viral load < 50 HIV-1 RNA copies/ml and $CD4^+$ T cells >400 cells/mm^3 for at least 12 and 6 months before inclusion, respectively. An in-house algorithm based on the Fiebig classification of HIV infection[43,44] and each participant's available HIV-1 diagnostic tests were used to calculate the estimated date of HIV-1 acquisition for each individual.

Before inclusion, all participants signed an informed consent previously reviewed by a local Community Advisory Board. The study was approved by the institutional ethical review board of HUGTIP (Reference Nr AC-15- 108-R) and by the Spanish Regulatory Authorities, and was conducted in accordance to the principles of the Helsinki Declaration and local personal data protection law (LOPD 15/1999).

For safety purposes, participants were randomized (2:1) in three sequential recruitment blocks after blinded safety reports were approved by an external SMC. A sentinel group of three participants (2 vaccine and 1 placebo recipients) was first enrolled, one participant was randomized per day and was monitored 24h after each vaccination (Group 1) to allow for the next sentinel participant to be vaccinated. The rest of the participants were part of the non-sentinel groups: Group 2 (n=12) and Group 3 (n=30). After completion of first vaccination regimen (DDDMM/placebo), all 45 participants were offered to participate into a second phase of the study, which included a booster vaccination regimen with CCM or placebo (while maintaining the same treatment allocation from the initial regimen) and into an ATI period of 24 weeks. Between DDDMM/placebo and CCM/placebo phases of the study, participants were kept on suppressive ART and performed clinical follow-ups every 12 weeks (Roll-Over period).

Criteria to proceed to ATI and resume ART: Eight weeks after the last vaccination (DDDMM-CCM or placebo) participants underwent an ATI of up to 24 weeks of duration if they had: i) received all vaccinations, ii) maintained pVL <50 copies/ml and $CD4^+$ T cells >400 cells/mm^3, and iii) there was no evidence of active syphilis, hepatitis B or hepatitis C infections. Before the ATI start, HIV seronegative participant's sexual partners were offered PrEP through a trial-specific PrEP-provision program. During the ATI, weekly visits were performed at HUGTIP, Badalona or at BCN-Checkpoint, Barcelona following participant's convenience. During the COVID19 pandemic, remote visits and home-based blood draws were implemented. Criteria to resume ART included: a single pVL > 100,000 copies/mL, pVL >10,000 and ≤ 100,000 copies/mL for 8 consecutive weeks, $CD4^+$ T cells <350 cells/mm^3 in two consecutive determinations, development of a ≥ Grade 3 ARS, at participant's request or investigator criteria. As part of investigator criteria, active surveillance for STI was performed during the ATI and, if suggestive of unprotected sex with partners with unknown HIV status and/or HIV negative partners not taking PrEP, ART was recommended to prevent HIV transmission. All participants off ART after 24 weeks of ATI were offered to resume ART except if pVL <2,000 copies/ml. These participants were invited to participate in an ATI-extension protocol (NCT04385875). Criteria for ART resumption during the ATI-extension phase included one determination of pVL >100,000 copies/ml or pVL>2,000 copies/ml for 8 consecutive weeks. Psychological assessments of the impact of the ATI on emotional and sexual sphere were evaluated using trial-specific questionnaires by clinical psychologists at the HIV unit before entering the ATI, 12 weeks after the ATI, 4 weeks after ART was resumed and at participant's request. Participants were followed 4 and 12 weeks after ART was resumed. The Protocol and a list of amendments to the protocol are available as Supplementary files S1 and S2.

Study vaccines. HTI immunogen is a chimeric protein sequence (total length of 529 aa) that was designed based on human immune reactivity[45] that includes 26 regions in HIV-1 Gag (45%), Pol (44%), Vif (8%), and Nef (3%) proteins identified in these analyses that (i) were preferentially targeted by participants with low viral loads and largely independent of beneficial HLA class I genotypes, (ii) turned out to be more conserved than the rest of the proteome, and (iii) elicited responses of higher functional avidity and broader variant cross-reactivity than responses to other regions[46].

DNA.HTI vaccine (D) is a circular and double stranded deoxyribonucleic acid (DNA) plasmid vector of 5,676 base pairs derived from the pCMVkan expression vector backbone expressing the codon-optimized HTI gene, preceded by the human Granulocyte-macrophage colony-stimulating factor (GM-CSF) signal peptide for better secretion[47]. The DNA.HTI DS is manufactured, quality-control tested and released in accordance with the requirements of good manufacturing practice (cGMP) by the Clinical Biotechnology Centre (CBC), Bristol Institute for Transfusion Sciences, University of Bristol, UK.

MVA.HTI vaccine (M, Modified Vaccinia Virus Ankara) is a live, attenuated recombinant vaccinia (pox) virus attenuated by serial passages in cultured chicken embryo fibroblasts (CEF) that contains six large deletions from the parental virus genome[48]. The size of MVA.HTI after the insertion of a transgene coding for the HTI insert is estimated to be approximately 179.6 kbp. The production is carried out by the German company IDT Biologika an all the preparation, verification of the genetic stability and MSV and WSV storage is done at IDT under cGMP conditions and according to EU regulations.

ChAdOx1.HTI vaccine (C)- is a replication-defective recombinant chimpanzee adenovirus (ChAd) vector based on a chimpanzee adenoviral isolate Y25[49] that encodes the HTI sequence. ChAdOx1.HTI was derived by sub-cloning the HTI antigen sequence into the generic ChAdOx1 BAC. The plasmid resulting from this sub-cloning (pC255; 40,483 bp) was linearized and transfected into commercial HEX293A T-REx® cells to produce the vectored vaccine ChAdOx1.HTI.ChAdOx1.HTI batch for non-clinical use have been performed at the University of Oxford (UK), whereas large scale amplification and purification of ChAdOx1.HTI have been performed at ReiThera/Advent (Italy) according to cGMP.

Objectives: The primary objective of the study was to evaluate the safety and tolerability of HIV-1 vaccines DNA.HTI, MVA.HTI and ChAdOx1.HTI administered intramuscularly as part of heterologous prime-boost regimen (DDDMM - CCM) in early treated HIV-1 positive individuals. Secondary objectives included i) to evaluate the immunogenicity of DDDMM and CCM, ii) to evaluate whether vaccination was able to prevent or delay viral rebound, induce post-rebound viral control, and/or prevent or delay the need for resumption of antiretroviral therapy during an ATI and iii) to assess the safety of the ATI period. Further immune (Flow cytometry, viral inhibition assay) and viral evaluations (viral reservoir, autologous HIV-1 sequence and replicative fitness) were conducted as exploratory analysis. Post-hoc univariate and multivariate regression models were performed to explore potential correlates of virus control during ATI.

Safety. Safety was assessed by an analysis of local and systemic reactogenicity and laboratory data. All solicited local and systemic adverse events (AEs) were recorded during 7 days after administration of each investigational medicinal product using a "Participant reactogenicity diary card". Unsolicited AEs and SAEs were recorded at any point during the study. AEs were graded according the Division of DAIDS table for grading the severity of adult and paediatric adverse events, Version 2.1. [March 2017]. Throughout the study, AEs were analyzed by period: from screening to ATI start and by DDDMM/CCM or placebo; during ATI and after ART resumption. The primary safety endpoint of the study was the proportion of participants who develop a Grade ≥ 3 AEs (including SAE) related to the IMP

administration. AEs were specified as related or unrelated to the IMPs by the investigator. Per the Manual for Expedited Reporting of Adverse Events to DAIDS (Version 2.0, January 2010), AEs were reported as related if there was reasonable possibility that the AE may be related to the study agent(s) suggested by a plausible, reasonable time sequence existed in relation to administration of the drug, the observed manifestation coincided with the known adverse reactions profile of the implicated drug, the event could not be or unlikely be explained by a concurrent disease or by other drugs or chemical substances. If there was not a reasonable possibility that the AE was related to the study agent(s), the AE was reported as unrelated.

Safety Monitoring Committee and Risk-Mitigation plan during COVID-19 pandemic. An SMC formed by three external experts in pharmacovigilance and HIV vaccine trials plus four non-voting sponsor representatives reviewed all blinded safety data from the study at pre-specifiedtime points (i.e. before progressing recruitment groups and every 3 months thereafter). The SMC also reviewed and approved a risk-mitigation plan established to minimize the impact of the COVID-19 pandemic on the conduct of the trial. This plan included: weekly ATI assessments with home-based blood draws by PPE-protected personnel and remote visits via phone; taxi service for on-site visits; 24h/7d phone availability for reporting any COVID-19 symptoms; SARS-CoV-2 PCR testing before any IMP dosing; and provision of ART by courier. The SMC virtually met weekly from 16th March 2020 to 28th May 2020 to review all blinded safety and laboratory data, and decisions on whether continuing with the trial were based on the evolving situation of the local epidemic, site capacity, and a case-by-case discussion. New ICF versions with emerging information on COVID-19 were also developed and reviewed by the institutional ethical review board of HUGTIP.

High-resolution HLA-A, -B and -C typing. The QIAsymphony DNA kit (Qiagen) was used for genomic DNA extraction. Genomic DNA was genotyped at screening for HLA class I molecules (HLA-A, HLA-B, and HLA-C genes) at high resolution at the Histocompatibility and Immunogenetics Laboratory (www.bancsang.net). Briefly, three loci were genotyped simultaneously by an in-house multiplex long-range PCR (LRPCR). The library was prepared (enzymatic fragmentation, adapter ligation, and barcoding) from the PCR pools using the NGSgo kit (GenDx) according to the manufacturer's instructions. The final denatured library was sequenced using a NextSeq or MiSeq sequencer (Illumina, San Diego, California, USA). HLA class I genotype determination was performed with NGSengine 2.9.1 software (GenDx) using the IMGT database as a reference.

CCR5-Δ32 genotyping. DNA was extracted from cryopreserved PBMCs stored from Roll-over phase timepoints from participants entering the ATI (n=41). DNA samples were amplified using fluorescent

PCR in a 9 700 Gene Amp® PCR System or 2 720 Thermal Cycler (Applied Biosystems) as described [50]. The forward (TTCATTACACCTGCAGCTCTC) and reverse (FAM™- CCTGTTAGAGCTACTGCAATTAT) primers used produced a 270-bp product for the CCR5-Δ32 allele and a 302-bp PCR product for the CCR5-WT allele. After amplification, 0.5 µL of PCR products were mixed in a 1:10 dilution with 24 µL of Hi-Di™ Formamide (Applied Biosystems) and 0.7 µL of Gene Scan™-500 ROX™ Size Standard (Applied Biosystems) and denatured at 94 °C for 5 min. The capillary electrophoresis was carried out in a 3130xlGenetic Analyzer (Applied Biosystems) and samples were analyzed with GeneMapper software (Applied Biosystems).

Sequencing. Whole genome deep sequencing of the HIV-1 genome, including *gag, pol, vif* and *nef* genes was performed using Illumina® NexteraXT protocol and MiSeq platform with 300 bp paired-end sequencing length. Raw sequencing data were analysed through PASeq v 1.14 (www.paseq.org[51]). In brief, quality filter and adapter trimming was performed using trimmomatic[52]. High quality sequences were aligned against HXB2R reference using Bowtie2[53]. Consensus sequence at 20% frequency threshold was called using samtools[54] and a multiple alignment including all sequences was generated using MAFFT[55]. For each sample-specific consensus nucleotide sequence, subtyping was performed using COMET online tool[56], and Tamura-Nei nucleotide and Jones-Taylor-Thornton (JTT) amino acid distances vs HXB2R and HTI sequences, respectively, were calculated using R::phangorn package[57]. The number of mismatches (hamming) vs HTI sequence was also calculated for all segmented and aggregated at the protein level. The percentage difference (%AA.mm vs HTI) was calculated over the total length of the segment correcting for uncovered position in each samples. Group comparisons were performed using Mann-Whitney t-test.

IFN-γ- ELISpot assay. Total HTI and HIV-1-specific T cells were assessed ex vivo using freshly isolated PBMC with an IFN-γ-detecting enzyme-linked immunoabsorbent spot assay (ELISPOT IFN-γ Mabtech kit) as previously described[58]. 15-mer peptides overlapping by 11 amino acid were combined into 10 pools spanning different HIV-1 proteins/subproteins of 7-22 peptides per pool corresponding to the HTI vaccine insert (P1-P10, total n = 111 peptides, ThermoFisher) and 8 pools of 62–105 peptides per pool spanning the rest of the HIV-1 viral protein sequences (OUT P1-P8, total n = 637 peptides, obtained through the NIH AIDS Reagent Program). All peptides pools used in fresh ELISPOTS were tested in duplicates with a final concentration of individual peptide of 1.55 µg/ml. Medium only was used as no-peptide negative control in quadruplicate wells. Positive controls included two peptide pools covering lytic (n=16) and latent (n=36) Epstein- Barr viral proteins (1.55 µg/ml, ThermoFisher), PHA (50µg/ml, Sigma) and a CEF peptide pool (2 µg/ml) consisting of 32 previously defined human CD8+ T-cell epitopes from cytomegalovirus, Epstein- Barr virus and influenza virus (Pantec). Spots were

counted using an automated Cellular Technology Limited (C.T.L., OH, USA) ELISPOT Reader Unit. The threshold for positive responses was set at ≥50 SFC/10^6 PBMC (5 spots per well), > the mean number of SFC in negative control wells plus 3 SD of the negative control wells, or > 3× the mean of negative control wells, whichever was higher.

Mapping of HTI-specific responses. IFN-γ ELISPOT assays using 147 individual overlapping peptides covering the entire HTI sequence were performed in *in-vitro* expanded T cells. Participants' cryopreserved PBMCs obtained at baseline (Week 0) and after DDDMM (Week 24) and CCM or placebo vaccinations (Week 28) were expanded using an anti-CD3 mAb (12F6) and kept in culture until sufficient cell numbers were reached for each timepoint[59]. Two consecutive overlapping peptides were considered one individual HTI response and the highest magnitude of the sequential responses was taken as the magnitude for each identified response. The results were expressed as the number of positive responses to individual peptides as well as distribution among the different 8 HIV subprotein regions covered by HTI: Vif-Nef, Pol-Int, Pol-RT, Pol-Prot, Gag-p2p7p1p7, Gag-p24 and Gag p17.

Intracellular Cytokine Staining (ICS) Assay. Cryopreserved PBMCs from week 28 (4 weeks after completion of last series of vaccinations, DDDMM-CCM) were used for the stimulation with 4 pools of 9-43 peptides per pool spanning p17, p24/p15, Pol and Vif/Nef regions included in the HTI vaccine insert. Peptides were added at a final concentration of 5ug/ml of each peptide in the presence of both, 1.4µg/ml of anti-CD28 (BD Bioscience) and 1.4µg/ml anti-CD49d (BD Bioscience). As positive controls for the assay, cells were cultured alone in the presence of 1) anti-CD3/28 Dynabeads (Thermo Fisher Scientific) according to manufacturer´s instructions or 2) 10ng/ml PMA (SIGMA) and 1µM Ionomycin (SIGMA). Cells stimulated with only anti-CD28 and anti-CD49d antibodies or with DMSO were used as the negative controls. Stimulated cells were incubated for 6 h at 37˚C in 5% CO2, in the presence of 4µl of monensin (GolgiStop, BD Bioscience). After 6 hours of stimulation, cells were incubated with Live/Dead fixable Violet Dead cell stain kit (Invitrogen), for exclusion of dead cells, along with the exclusion of monocytes and B cells by including in the dump channel anti-CD14 and anti-CD19 antibodies. Surface markers of T cell linage (CD3, CD4 and CD8), follicular T cells (CXCR5 and PD1), T cell phenotype (CD45RA and CCR7), T cell activation (CD69 and HLADR) and T cell exhaustion (TIGIT, PD1) were included as well. Cells were fixed and permeabilized using the Cell Fixation and Cell Permeabilization Kit (Invitrogen) and intracellularly stained for INF-γ, GrazymeB, IL-2 and TNF-α. Details on antibodies used can be found in Reporting Summary. Cells were resuspended in PBS supplemented with 1%FBS and acquired on a LSR Fortessa flow cytometer (BD, Unidad de Citometria, IGTP) and analyzed using FlowJo. Gating strategy is shown in Extended data Fig. 7. When needed for variably expressed antigens, fluorescence minus one (FMO) was included to define boundaries

between positive and negative populations. At least 100,000 total events were recorded. The frequencies of cells that produce all possible combinations of intracellular cytokines were calculated using Boolean gating function of the FlowJo software. Data were reported after background subtraction (from the unstimulated negative control), and HTI-specific responses were defined as the as the sum of the specific population for each of the four HTI peptide pool stimulations.

In vitro viral suppressive capacity (VIA assay). CD8+ T-cell mediated viral inhibition capacity was measured at 1:1 and 1:10 CD8-effector to CD4-target ratios as previously described[60,61]. Autologous $CD4^+$ cells were obtained as targets from samples before vaccination where $CD8^+$ cells were depleted by magnetic bead separation (MACS Milteny Biotec). $CD8^+$-depleted cells ($CD4^+$-enriched fraction) were stimulated with PHA for 3 days and then infected by spinoculation with HIV-1 BAL and IIIB laboratory-adapted strains and autologous HIV-1 viruses at a multiplicity of infection (MOI) of 0.001. HIV-infected cells were cultured in triplicates in R10 medium with 20 U/ml of IL-2 in 96-well round-bottomed plates, alone or together with effector $CD8^+$ T cells obtained by positive magnetic bead separation the same day from an additional vial of cryopreserved PBMCs from baseline and after DDDMM (Week 24) and CCM or placebo vaccinations (Week 28). Viral replication was measured as the production of HIV-1 antigen p24 in culture supernatants (pg p24/mL) at day 5 of co-culture using Innogenetics p24 Elisa kit, and inhibition was expressed as a percentage with respect to the positive control of each virus (i.e., infection in the absence of $CD8^+$ T cells).

Total and Intact proviral HIV-1 DNA. To distinguish deleted and/or hypermutated proviruses from intact proviruses, total and intact proviral (IPDA) HIV-1 DNA copies in $CD4^+$ T cells were measured at screening and ATI start in extracts of lysed $CD4^+$ T cells by digital droplet PCR (ddPCR) as previously described[62]. Samples from 41 participants that entered into the ATI period were processed at Accelevir Diagnostics, Baltimore, US. The DNA Shearing Index (DSI) was calculated and values for intact and defective proviruses were normalized to copies per 10^6 input cells (determined by RPP30, the gene encoding Ribonuclease P protein subunit p30) and adjusted for shearing using the DSI. Results were expressed as HIV-1 DNA copies (counts)/10^6 $CD4^+$ T cells.

Viral fitness of participants' autologous HIV-1 viruses. Viral replication capacity of autologous HIV-1 viruses was measured for 38 out of the 41 participants that entered into the ATI period. For isolation of autologous HIV-1 viruses, CD4-enriched fraction of cryopreserved PBMCs stored at HIV-1 diagnosis pre/or within first weeks of ART initiation were thawed and co-cultured with CD8-depleted PBMCs previously activated from 3 different healthy donors until HIV-1 was collected from supernatants. To determine viral replication kinetics, a pool of PBMCs from 3 healthy donors, previously stimulated with

20 U/mL of IL-2 and PHA for 3 days, were infected by spinoculation at a multiplicity of infection (MOI) of 0.001. HIV-1 antigen p24 was measured in culture supernatants (pg p24/mL) using a commercial ELISA kit from Innogenetics at days 0, 3, 4, 5, 6, and 7 post-infection and replication capacity was calculated by fitting a linear model to the log-transformed p24 data during the exponential growth phase. Uninfected cells and infected with laboratory-adapted CCR5- and CXCR4-tropic viruses (HIV-1_{NL43}, HIV-1_{BaL}, and HIV-1_{IIIB} isolates) in the presence and absence of the antiretroviral AZT, were used as reference values or controls.

Statistics. There was no power calculation for this study. The sample size was proposed to provide preliminary safety information on the vaccine regimen (primary objective). As a means to characterize the statistical properties of this study for the safety primary endpoint, in terms of the chances of observing an AE, 30 participants in the active group provided a high probability (78.5%) that this study would observe at least 1 event if the event occurred in the population with a true rate of 5%.

Time to viral load detection was calculated from the ATI start date to the date of first occurrence of pVL $\geq$ 50 copies/ml and time off ART was calculated from the ATI start date to the date of ART resumption. Participants who prematurely resumed ART due to COVID-19 related reasons were not censored for the survival analysis. The time-to-event was derived using number of days between ATI start date and date of event expressed in weeks (number of days/7). The Kaplan–Meier estimator was used to describe time to ART resumption and survival functions were compared using log-rank test. Differences of medians between groups were compared using Mann-Whitney test and Fisher test, when corresponding. Spearman rho were used for correlations. All tests were two-sided, unadjusted for multiple comparisons, with 5% error rate. Post hoc univariate logistic regression models (the list of the considered covariates can be seen at Extended data Table 5) were considered to select the covariates with $p<0.25$ to be included in the multivariate models. All selected covariates were analyzed for possible multicollinearity. Considering the final selected covariates multivariate logistic regression models were adjusted for the binary outcome of time off ART >=12 weeks versus <12 weeks. Analyses were performed using R project 3.6.2 (https://www.r-project.org/) and GraphPad Prism version 9.1.2 for Windows (GraphPad Software, https://www.graphpad.com). Preprocessing of flow cytometry data was performed using both FlowJo software version 10.6 and imported into Pestle2/ SPICE software v5.35 (Vaccine Research Center, NIAID/NIH, Bethesda, MD, USA) for graphical representation. Polyfunctional bar plots per treatment group were compared using Mann-Whitney test per row, with individual ranks computed for each comparison. Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was used to control for false discovery rate. All analyses performed matched the prespecified statistical analysis plan (AELIX002-SAP, version 2, from 10/07/2020).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Deep sequencing raw data obtained from sequencing have been deposited in GenBank (accession PRJNA751460). Requests for access to the study data can be submitted through the Yale Open Data Access (YODA) Project site at http://yoda.yale.edu.

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A full list of members of the AELIX-002 Study Group appears in the Supplementary Data S3

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